AD_____

GRANT NUMBER DAMD17-96-1-6285

TITLE: Control of the Mammary Cell Cycle Clock By Estrogen and Progesterone

PRINCIPAL INVESTIGATOR: Robert A. Weinberg, Ph.D.

CONTRACTING ORGANIZATION: Whitehead Institute for Biomedical Research
Cambridge, Massachusetts 02142

REPORT DATE: August 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 1998	Annual (15 Jul 97 - 14 Jul 98)

4. TITLE AND SUBTITLE Control of the Mammary Cell Cycle Clock by Estrogen and Progesterone

5. FUNDING NUMBERS
DAMD17-96-1-6285

6. AUTHOR(S)
Robert A. Weinberg, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Whitehead Institute for Biomedical Research
Cambridge, Massachusetts 02142

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200)*

Growth and development of the mammary gland are controlled by the female hormones estrogen and progesterone. These hormones affect breast cancer growth and are targets for hormonal therapy. For this reason, it is important to understand the role of these hormones in the proliferation of mammary epithelial cells. We have characterized how these hormones may be interacting with the cell cycle machinery. The cell cycle machinery orchestrates the events required for cells to proliferate. It is composed of cyclins and their catalytic partners known as cyclin-dependent kinases. We have addressed how estrogen and progesterone affect expression and activities of these cell cycle molecules. We have shown that estrogen can induce cyclin D1 expression and activate cyclin E-cdk2 complexes in breast cancer cells. We have also investigated the relationship between progesterone and cyclin D1 using mice lacking either cyclin D1 or the progesterone receptor. Both strains have a defect in mammary gland development. We postulated that cyclin D1 may be regulated by progesterone during pregnancy. However, mammary glands lacking cyclin D1 responded to progesterone like normal mammary glands.

In conclusion, we have studied the role of estrogen and progesterone in regulating the cell cycle of mammary epithelial cells. Understanding the growth-regulatory mechanisms affected by these hormones may lead to new ways of breast cancer therapy.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	19
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

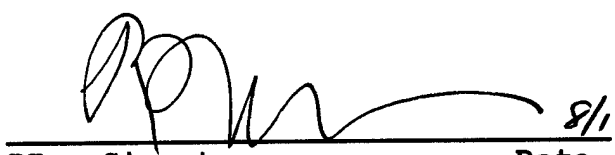
AW In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

AW In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

AW In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

AW In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.



8/12/98

PI - Signature Date

TABLE OF CONTENTS

Annual Report
Laboratory of Robert A. Weinberg
Whitehead Institute for Biomedical Research
Cambridge, MA 02142

INTRODUCTION

The design of the experiments described here is driven by the notion that the pathogenesis of human breast cancer can only be understood once the normal morphogenetic processes in the breast have been elucidated at the molecular level. Such morphogenesis is most easily studied in the context of the mouse breast, where several experimental advantages obtain. These include i) the ability to explant mammary epithelial cells from one donor animal and engraft them into a host in which they re-form normal mammary ductal trees and alveoli; ii) the ability to manipulate the mammary epithelial cells ex vivo, including the ability to introduce ectopically expressed genes via retrovirus vectors into these cells; and iii) the availability of mutant mice strains that lack one or another of the genes critical to mammary development, including for example, those specifying the estrogen, progesterone or prolactin receptors (1,2,3). For these reasons, many of our experiments over the past year have focused largely on the use of mouse models of mammary development as described below.

A second line of work has derived from our study of the estrogen receptor (ER) and the mechanism by which it drives mammary epithelial cell proliferation. Estrogen treatment of ER-positive mammary carcinoma cells results in the induction of cyclin D1. Ectopic expression of the ER in previously ER-negative carcinoma cells and keratinocytes does not lead to cyclin D1 production upon estrogen treatment. This has led us to hypothesize that there are two major mechanisms leading to formation mammary carcinomas. These models have been tested and are being tested in the experiments described below.

In attempting to describe the pathogenesis of ER-negative human mammary carcinomas, we have taken note of the fact that these often express high levels of the HER2/neu receptor, the receptor for heregulin(HRG) (4). HRG is normally elaborated by the mammary stroma during alveologenesis, and is likely to act upon the epithelial cells, driving their proliferation through its ability to evoke production of cyclin D1 in these cells. This process of alveologenesis is also correlated temporally with the presence of high levels of prolactin of pituitary and placental origin, which likely provokes this process through its ability to act on mammary epithelial cells (MECs). Moreover, our work has led us to realize that the phenotypes of breast development in cyclin D1-negative and prolactin receptor-negative mice are remarkably similar, being blocked just prior to alveologenesis.

Together, these observations have led us to the following mechanistic models of morphogenesis and tumorigenesis. During alveologenesis, prolactin impinges on prolactin receptor-positive epithelial cells, inducing them to elaborate a paracrine factor (perhaps a Wnt protein) that in turn stimulates nearby stromal cells to produce HRG. The latter then acts back on MECs, stimulating them to proliferate via its ability to induce cyclin D1 production in them. During the pathogenesis of ER-negative mammary carcinomas, we hypothesize that certain MECs acquire the ability to make their own prolactin, resulting in the formation of an autocrine signaling loop that results in the constitutive production of a paracrine factor(s) that elicit(s) HRG production in nearby stroma; the resulting HRG then stimulates MEC proliferation, resulting in localized ductal hyperplasia that is dependent on ongoing, intimate interaction with the stroma. Subsequently, during tumor progression, some of these hyperplastic cells overexpress their HER/neu receptor, causing its ligand-independent firing and thereby liberating these cells from stromal dependence and creating a ductal carcinoma in situ.

In the case of ER-positive tumors, we imagine a quite different scenario to be operating. In this instance, work of others has indicated that the sole function of estrogen and the ER in the mammary epithelium is to induce production of the progesterone receptor. Accordingly, estrogen is not a direct mitogen for normal mammary epithelial cells. We hypothesize that it fails to act in a mitogenic fashion because the signaling pathway between the ER and cyclin D1 is not operative in normal MECs. Such a connection becomes established, following our model, when the AIB-1/RAC3 co-activator protein of the ER becomes ectopically expressed in ER-positive MECs. Such ectopic expression enables MECs to take advantage opportunistically of ambient estrogen, using it as a mitogen to drive their proliferation. These two models of breast development and mammary carcinoma pathogenesis are being tested in the experiments described below.

BODY

Project 1: Wnt-4 and mammary development: ductal branching and alveologenesis

As described above, we hypothesize that paracrine signaling, provoked by prolactin, from epithelial cells to stromal cells is critical for alveologenesis. Yet other evidence, not described here, suggests analogous paracrine signaling provoked by progesterone is also important for ductal branching and the initiation of alveologenesis. We speculate that this paracrine signaling is mediated by various Wnt proteins and accordingly have initiated detailed examination of the behavior of various Wnt proteins and their effects on mammary epithelial and stromal cells.

The Wnt-1 gene was originally identified as a frequent integration site for the Mouse Mammary Tumor Virus (5). Wnt-1 is one of a group of more than a dozen related proteins that appear to act as paracrine signals that mediate differentiation and proliferation of cells. Overexpression of Wnt-1 in the mammary epithelium leads to increased branching and alveolar budding in the

gland and to carcinoma formation within about 6 months. Although the expression of Wnt-1 has strong morphogenetic effects in the mammary gland, Wnt-1 itself has never been found to be expressed in the breast under physiologic conditions. However, other related Wnt proteins are indeed expressed in a developmentally regulated fashion. In particular, Wnt-4 is expressed in the mammary epithelium and is upregulated during puberty and early pregnancy (6,8) when increased branching and budding occur. Yet others (7) demonstrated that overexpression of Wnt-4 in the mammary epithelium by means of retroviral vector infection causes sidebranching and alveolar formation in virgin mice, suggesting that Wnt-1 might actually be activating a physiologic pathway normally controlled by Wnt-4.

To gauge the importance of Wnt-4 in mammary gland development, we have collaborated with the laboratory of A. McMahon at Harvard in whose lab were generated germline mutant mice lacking functional Wnt-4 gene copies. Any effects of Wnt-4 loss on mammary development could not directly be assessed, since these mice die at birth from kidney failure. For this reason, we resorted to isolating mammary buds from both wnt-4$^{-/-}$ and wild type (wt) embryos and implanting these into the cleared fat pads of three week-old recipients. An extensive series of such transplant experiments showed that ductal branching occurred normally in virgin recipients. However, when the recipients were analyzed during the first three quarters of the pregnancy, the epithelium lacking Wnt-4 showed retardation in sidebranching and alveolar formation compared to the contralaterally implanted wt epithelium. By the end of pregnancy, both implants showed comparable degree of development. This indicates that Wnt-4 is important for sidebranching and alveolar formation during the first three quarters of pregnancy, but later on, other Wnt proteins, may compensate for its absence. Included among these are the Wnt-5a and -5b and -6 proteins, the expression of which is upregulated after Wnt-4 during pregnancy (8).

Our earlier work (last year's report) along with that of others provided strong indication that progesterone, acting on progesterone receptor-positive epithelial cells, contributes importantly to ductal branching (9,10). In particular, we showed previously that progesterone acts on the mammary epithelium in a paracrine fashion to induce sidebranching and allow for alveolar formation. The present observations concerning Wnt-4 implicate it as well in the process of ductal branching and alveologenesis, making it an attractive candidate as a downstream mediator of progesterone action. Accordingly, we are currently testing whether progesterone is able to induce Wnt-4 expression in vivo in the mammary epithelium by injecting mice with this hormone, and in vitro by stimulating cells of the T47D progesterone-responsive breast cancer cell line, looking at the expression levels of Wnt-4 by Northern analysis.

Project 2: Prolactin, Cyclin D1 and Alveologenesis

As described above, several lines of evidence indicate that cyclin D1, prolactin, and heregulin are all intimately involved in alveologenesis. According to our own thinking, prolactin treatment of epithelial cells may cause them to release a paracrine signal to the nearby stroma, inducing the latter to elaborate heregulin, which in turn elicits alveologenesis by activating cyclin D1 expression in nearby epithelial cells. To test whether heregulin is indeed downstream of the prolactin receptor and upstream of cyclin D1, as depicted in this model, we would like to infect primary mammary epithelial cells derived from cyclin D1-negative ($D1^{-/-}$) mice and prolactin receptor-negative ($PRLR^{-/-}$) mice with a retrovirus that causes ectopic expression of heregulin (HRG). The infected cells will then be used to reconstitute cleared fat pads. If the model as presented is correct, the ectopically expressed HRG should be able to rescue the phenotype of the $PRLR^{-/-}$ cells but not that of cells deriving from cyclin $D1^{-/-}$ mice. Infected cells can be visualized by blue stain, since the viral construct encodes an IRES-beta-geo gene in addition to heregulin.

Over the past six months, we have been optimizing the infection of primary mammary epithelial cells and the injection techniques using a b-galactosidase-expressing retrovirus and blue staining of infected cells and injected tissue, the latter performed 8 weeks after implantation of infected cells into cleared fat pads. We have gradually improved the conditions and have now obtained a series of reconstituted breasts that have segments expressing the b-galactosidase gene. We have subsequently been successful in obtaining expression of a wnt1-IRES-beta-geo virus in spite of the fact that the viral titers are lower than those obtained with the vector expressing only b-galactosidase, and have now successfully reconstituted glands with heregulin-infected wt cells.

In order to dissect further the biochemical connection between the prolactin receptor and cyclin D1, we have decided to look at the phenotype of mammary epithelium lacking STAT5a and b, which are immediately downstream of the prolactin receptor (11). These two transcription factors may mediate some of the alveologenic response elicited by prolactin. They may act directly by inducing cyclin D1 in mammary epithelial cells, or as we suspect, indirectly by allowing prolactin-treated epithelial cells to release a paracrine factor that induces HRG from nearby stromal cells; once released, according to our thinking, the HRG may proceed to induce cyclin D1 synthesis and alveologenesis in the breast tissue. We wish to know whether these two STATs, which appear to act redundantly, are needed for both the proliferative and the differentiative response to prolactin. Because STAT5a/b $^{-/-}$ mice fail to become pregnant (11), their mammary phenotype could not be assessed directly. Consequently, we have generated a small breeding colony and set up several transplantation experiments in which their breast tissue will be engrafted into the cleared fat pads of wild type hosts that in turn will be induced to become pregnant. Our analysis of the behavior of the resulting epithelial grafts should prove revealing about the role of the STATs in mediating the prolactin response.

Project 3: Wnts and mammary morphogenesis

We believe that paracrine signals flowing from the mammary epithelium to the stroma are important for inducing the latter to elaborate the important morphogens, HRG and hepatocyte growth factor (HGF). According to our thinking, the latter then proceed to induce morphogenetic steps such as ductal elongation and branching and alveologenesis in the epithelium. We believe, moreover, that the Wnt proteins are excellent candidates for these paracrine factors conveying the epithelium-to-stroma signals. As described, overexpression of certain wnt genes in the mouse mammary gland is tumorigenic and has been observed in some human breast cancers. In the mouse mammary gland, six different Wnt proteins are expressed at defined times during development and four of these (Wnts -4, -5A, -5B and -6) are strongly induced during pregnancy (12) . However, the precise role of these Wnts in mammary gland development and cancer remains unclear.

We are currently testing the hypothesis that specific Wnt proteins are mediators of epithelial/stromal communication in the mammary gland in response to hormonal stimulation. More specifically, we are interested in whether Progesterone (P) or Prolactin (Prl), the two major pregnancy-associated hormones, causes the expression of certain Wnt genes in mammary epithelial cells (MECs), the products of which act on neighboring mammary stromal fibroblasts causing the production of the heregulin (HRG) growth factor during pregnancy. HRG expression is restricted to the mammary stroma but HRG elicits its effects on the epithelial cells expressing its receptors, the erbB-3 and erbB-4 receptor tyrosine kinases.

To test this model, we are first testing whether P or Prl is able to induce the expression of specific wnt genes in P- or Prl-responsive MEC cell lines by Northern analysis and RT-PCR. Independent of this, we have constructed a series of retrovirus vectors expressing each of the different Wnt proteins known to be expressed in the mouse mammary gland. We are introducing these into primary mammary fibroblasts and a series of other types of fibroblasts to examine whether HRG expression is being induced in response to one or another ectopically expressed wnt. Expression of the different Wnt proteins following retrovirus infection of several fibroblast lines has been confirmed by western immunoblotting with an antibody that recognizes an epitope tag on the C-terminus of each Wnt protein. These studies will hopefully begin to define a specific role for one or more of the Wnt proteins as mediators of epithelial/stromal communication during mammary gland development and cancer.

Project 4: Autocrine Prolactin Signaling and Breast Cancer Initiation

As described above, we believe that prolactin (Prl) induces (MECs) to elaborate a paracrine factor (perhaps a Wnt) that induces heregulin (HRG) production in the stroma. The latter then proceeds to drive epithelial cells proliferation and alveologenesis. This model suggests a mechanism by which certain estrogen receptor-negative carcinomas may be initiated. Thus, an epithelial cell may acquire the ability to make its own Prl rather than being dependent upon Prl elaborated by the pituitary or placenta. As a consequence, it may stimulate in an autocrine fashion its own Prl signaling pathway, causing this cell to release in a constitutive fashion the paracrine factor that is

responsible for inducing HRG production in the stroma. Once produced by the stroma, this HRG may then drive the proliferation of nearby epithelial cells, activating their cyclin D1 expression and resulting ultimately in ductal hyperplasia. Such hyperplastic growths may eventually progress to carcinomas in situ after amplifying their HER2/neu receptors, causing these receptors to fire in a ligand-independent fashion, thereby allowing hyperplastic epithelial cells to acquire an independence from stromal stimulation.

As one way of testing this model, we have begun to examine whether human mammary carcinoma lines do indeed elaborate paracrine factors that elicit HRG production in stromal fibroblasts. To do so, we have begun a series of experiments in which we are co-culturing a series of human mammary carcinoma cell lines, including the BT-474, CAMMA-1, MCF-7, MDA-MB-231, MDA-MB-453, SK-Br-3, T47D lines, with mouse mammary stromal fibroblasts, asking whether the latter respond to the carcinoma cells by inducing the production of mouse HRG, the latter being measured in RNase protection assays, by Northern blotting, and by RT-PCR (reverse transcription-polymerase chain reaction). We have recently prepared the appropriate probes and primers for these various assays. To extend these studies to interactions that may occur in vivo, we have also begun to implant human mammary carcinoma cell lines into the cleared mammary fat pads of RAG-1 immunocompromised female mice, asking whether the carcinoma cells are able to elicit HRG production from the stromal cells residing in the fat pads. As before, we are assessing mouse HRG production by Northern blotting, RNase protection assays, and by RT-PCR analysis.

As a further test of this model, we are infecting mouse mammary epithelial cells with a retrovirus vector that specifies Prl, thereby mimicking the state that we hypothesize exists in early human mammary carcinomas that have acquired the ability to synthesize their own Prl. We are testing the biological consequences of this infection in two ways. First, by co-culturing the Prl-producing epithelial cells with primary stromal fibroblasts, we hope to be able to detect HRG expression in the latter. Second, by introducing the Prl-producing epithelial cells into cleared fat pads of virgin females, we hope to detect HRG expression in the stromal cells of the fat pads. In both instances, we shall use, as before, Northern blotting, RNase protection assays, and RT-PCR to detect expression of the mouse HRG gene.

Project 5: Estrogen and the Pathogenesis of Estrogen-receptor positive mammary carcinomas

In an earlier report, we described our studies and those of others in which the effects of estrogen on cyclin D1 expression in human MCF-7 mammary carcinoma cells were described. Specifically, estrogen, acting via the estrogen receptor (ER), was able to activate expression of cyclin D1, thereby driving the growth of these cells (13, 14). In our ongoing work, we have attempted to understand how the ER is able to activate cyclin D1 expression.

In order to do so, we have ectopically expressed the ER in ER-negative MDA-231 human mammary carcinoma cells, as described previously. A series of clonal MDA-231-derived cells lines ectopically expressing the ER has been isolated. In each case, application of estrogen to these cells has failed to elicit cyclin D1 expression. We also have varied the conditions of estrogen treatment, by concomitantly starving cells of serum or amino acids or treating them with lovastatin; in all these instances, no cyclin D1 was induced. However, fetal bovine serum added to serum-starved MDA-231 cells was able to strongly induce cyclin D1 expression. We concluded that the presence of the ER per se is not sufficient in mammary carcinoma cells for the estrogen-dependent induction of cyclin D1 expression.

In light of the fact that MDA-231 cells are tumor-derived and may be aberrant in many respects, we sought to study ER function in a more normal cellular background. To do so, we infected HaCaT cells, a line of normal immortalized human keratinocytes, with an ER expression vector. A number of independent clones stably expressing different levels of ER have been isolated. Eleven clones were tested for estrogen-dependent cyclin D1 expression. None of these showed estrogen-dependent cyclin D1 expression while serum-dependent cyclin D1 expression was observed as expected.

Transient transfections of an estrogen-responsive promoter driving luciferase expression indicated the presence of a functional ER in several of the isolated HaCaT clones. Thus, as before, we concluded that ER per se is not sufficient to drive cyclin D1 expression in an estrogen-dependent fashion. This led us to conclude that other molecules or biochemical conditions, in addition to ER expression, must be present in order for estrogen to induce cyclin D1 expression and cell proliferation.

The most attractive candidates for such mediators of ER-to-cyclin D1 signaling are the recently isolated nuclear receptor coactivators. These molecules interact directly with nuclear receptors and are able to mediate ligand-dependent transcription. In particular, one of these co-activators, AIB-1/RAC 3, was recently found to be overexpressed in a series of human mammary carcinomas (15). Thus, it is possible that the signaling between the ER and the cyclin D1 promoter may be mediated through this molecule.

We are currently testing the possibility that AIB-1/RAC3 co-activator cooperates with ER to drive cyclin D1 expression. Transient transfection of AIB-1/RAC3 enhanced expression of an estrogen-responsive luciferase construct as reported by several laboratories. Preliminary studies with a cyclin D1 promoter-luciferase construct showed a dose-dependent increase in luciferase expression with increasing doses of AIB-1/RAC3 in MDA-231 cells containing ER. We are also characterizing the effect of ectopic AIB-1/RAC3 expression in the ER-positive HaCaT cells that we have created. To approach this, we have constructed a retrovirus vector that specifies AIB-1/RAC3; in addition, we have introduced the AIB-1/RAC3 cDNA into the mammalian vector pCI-neo for use in transfection experiments. In vitro transcription and translation of the introduced AIB-1/RAC3 genes confirmed that the expected

protein is being made by these new plasmids. In the near future, transfection and/or infection of AIB-1/RAC3 into MDA-231 or HaCaT cells expressing ectopic ER will allow us to test the possibility that expression of AIB-1/RAC3 is required for estrogen-dependent cyclin D1 expression.

Conclusions

1. The estrogen receptor requires additional signal transducers to enable it to activate cyclin D1 synthesis, thereby rendering estrogen mitogenic for mammary epithelial cells.

2. Wnt-4 plays a role during early but not late pregnancy in ductal branching and alveologenesis.

References

1. Korach, K.S., Couse, J.F., Curtis, S.W., Wasburn, T.F., Lindzey, J.K., Kimbro, K.S. Eddy, E.M., Migliaccio, S., Snedecker, S.M., Lubahn, D.B, Schomberg, D.W. and Smith, E.P. (1996). Estrogen receptor gene disruption: molecular characterization and experimental and clinical phenotypes. Rec. Prog. Horm. Res. 51,159-188.

2. Lydon, J.P., DeMayo, F.J., Funk, C.R., Mani, S.K., Hughes, A.R., Montgomery Jr., C.A., Shyamala, G., Conneely, O.M. and O'Malley, B.W. (1995). Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev. 9,2266-2278.

3. Ormandy, C.J., Camus, A., Barra, J., Damotte, D., Lucas, B., Buteau, H., Brousse, N, Babinet, C., Binart, N., and Kelly, P.A. (1997) Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. Genes Dev. 11,167-178.

4. Slamon, D.J., Clark,G.M., Wong, S.G., Levin, W.J., Ullrich, A., and McGuire, W.L. (1987). Human breast cancer:correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235,177-182.

5. Nusse, R. (1988). The int genes in mammary tumorigenesis and in normal development. Trends Genet 4, 291-295. Nusse, R., and Varmus, H. E. (1992) Wnt genes. Cell 69, 1073-1087.

6. Trevor Dale and Andy McMahon, personal communication.

7. Bradbury, J.M., Edwards, P.A., Niemeyer, C.C., and Dale, T.C. (1995). Wnt-4 expression induces a pregnancy-like growth pattern in reconstituted mammary glands in virgin mice. Dev. Biol. 170,553-63.

8. Gavin, B.J. and A.P. McMahon (1992). Differential regulation of the Wnt gene family during pregnancy and lactation suggests a role in postnatal development of the mammary gland. Mol. Cell. Biol. 12:2418-23.

Weber-Hall, S. J. Phippard, D. J. Niemeyer, C. C. Dale, T. C. (1994). Developmental and hormonal regulation of Wnt gene expression in the mouse mammary gland. Differentiation 57,205-214

9. Lydon, J.P., DeMayo, F.J., Funk, C.R., Mani, S.K., Hughes, A.R., Montgomery jr., C.A., Shyamala, G., Conneely, O.M. and O'Malley, B.W. (1995). Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev. 9,2266-2278.

10. Brisken, C., Vass, T. ,Park, S., Lydon, J.P, O'Malley, B.W., and Weinberg, R.A. (1998) A paracrine role for the epitheial progestrone receptors in mammary gland development. Proc. Nat. Acad. Sci. 95,5076-5081.

11. Liu, X, Robinson, G.W., Wagner, K.-U., Garrett, L, Wynshaw-Boris, A., and Hennighausen L. (1997). Stat5a is mandatory for adult mammary gland development and lactogenesis. Genes Dev. 11,179-186. Udy, G.B., Towers, R.P., Swell, R.G., Wilkins, R.J., Park, S,H., Ram, P.A., Waxman, D.J., Davey, H.W. (1997). Requirement of STAT5b for sexual dimorphism of body growth rates and liner gene expression. Proc. Nat. Acad. Sci. 94,7239-7244.

12. Bühler, T.A., Dale, T.C., Kieback, C., Humphreys, R.C., and Rosen, J.M. (1993). Localization and quantification of Wnt-2 gene expression in mouse mammary development. Dev. Biol. 155,87- 96.

13. Planas-Silva, M.D., and Weinberg, R.A. (1997). Estrogen-dependent cyclin E-cdk2 activation through p21 redistribution. Mol. and Cell. Biol., 17,4059-4069.

14. Prall, O. W. J., Sarcevic, B., Musgrove, E. A., Watts, C. K. W., and Sutherland, R. L. (1997). Estrogen-induced activation of Cdk4 and Cdk2 during G1-S phase progression is accompanied by increased cyclin D1 expression and decreased cyclin-dependent kinase inhibitor association with cyclin E-Cdk2. J. Biol. Chem. 272, 10882-10894.

15. Anzick, S. L., Kononen, J., Walker, R. L., Azorsa, D. O., Tanner, M. M., Guan, X. Y., Sauter, G., Kallioniemi, O. P., Trent, J. M., and Meltzer, P. S. (1997). AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science 277, 965-968.

Appendices

Reprint of:

Brisken, C., Vass, T. ,Park, S., Lydon, J.P, O'Malley, B.W., and Weinberg, R.A. (1998) A paracrine role for the epitheial progestrone receptors in mammary gland development. Proc. Nat. Acad. Sci. 95,5076-5081.

Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 5076–5081, April 1998
Developmental Biology

A paracrine role for the epithelial progesterone receptor in mammary gland development

CATHRIN BRISKEN*, SISSELA PARK*, TIBOR VASS*, JOHN P. LYDON†, BERT W. O'MALLEY†, AND ROBERT A. WEINBERG*‡

*Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142; and †Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030

Contributed by Robert A. Weinberg, February 13, 1998

ABSTRACT **Recently generated progesterone receptor (PR)-negative ($PR^{-/-}$) mice provide an excellent model for dissecting the role of progesterone in the development of the mammary gland during puberty and pregnancy. However, the full extent of the mammary gland defect in these mice caused by the absence of the PR cannot be assessed, because $PR^{-/-}$ mice do not exhibit estrous cycles and fail to become pregnant. To circumvent this difficulty, we have transplanted $PR^{-/-}$ breasts into wild-type mice, and we have demonstrated that the development of the mammary gland in the absence of the PR is arrested at the stage of the simple ductal system found in the young virgin mouse. Mammary transplants lacking the PR in the stromal compartment give rise to normal alveolar growth, whereas transplants containing $PR^{-/-}$ epithelium conserve the abnormal phenotype. Chimeric epithelia in which $PR^{-/-}$ cells are in close vicinity to PR wild-type cells go through complete alveolar development to which the $PR^{-/-}$ cells contribute. Together, these results indicate that progesterone acts by a paracrine mechanism on a subset of mammary epithelial cells to allow for alveolar growth and that expression of the PR is not required in all the cells of the mammary epithelium in order for alveolar development to proceed normally.**

The mouse provides a useful model to study mammary gland development. At the onset of puberty, a simple system of branching ducts begins growing out from the nipple area into a pad of fatty connective tissue that underlies the skin. During the luteal phase of the estrous cycles, the ductal system becomes more complex through the growth of side branches. Ductal side-branching becomes more extensive during early pregnancy, and subsequently alveolar bodies develop from these ducts, fill up the fat pad, and differentiate to become the sites of milk production.

The serum levels of the sex steroid progesterone are elevated during diestrus, the phase of luteal activity of the estrous cycle, and pregnancy. Moreover, experimental manipulation of the hormonal system has implicated this hormone as an essential stimulus required for the induction of ductal branching and for alveologenesis (1). However, the elucidation of the role of progesterone is complicated by the fact that, in the mammary epithelium, synthesis of the progesterone receptor (PR) depends on estrogen, the serum levels of which are also elevated during puberty and pregnancy. This has made it difficult to assess which developmental effects can be attributed to progesterone alone.

To dissect the role of progesterone from that played by estrogen, we generated mice lacking the PR by targeted inactivation of the PR gene in the mouse germ line (2). The mammary glands of the resulting young virgin $PR^{-/-}$ females show the same extent of ductal development as is seen in wild-type (wt) female mice (2). However, when wt and $PR^{-/-}$ virgin females were exposed to estradiol and progesterone, the wt breast tissue responded with side-branching and lobuloalveolar development, whereas the mammary glands of $PR^{-/-}$ females remained essentially unchanged. This suggested that PR is not required for initial ductal growth but is essential for subsequent side-branching and alveologenesis.

The administration of exogenous estrogen and progesterone, as was done in the above-described experiments and in a subsequent study extending this work (3), did not permit us to properly gauge the full spectrum of complex hormonal changes that occur during a normal pregnancy. During this period, the serum levels of a wide array of other hormones, including growth hormone, prolactin, placental lactogen, and adrenal steroids, are elevated. Moreover, the secretion of each of these hormones follows specific diurnal rhythms, and it is unlikely that injections of exogenous hormones achieve physiologic serum levels and correct local concentrations.

For these reasons, we resorted to transplanting $PR^{-/-}$ mammary tissues into wt animals that were subsequently impregnated. This allowed us to study the morphogenesis of the breast tissue in a hormonal environment that faithfully recapitulated that seen in pregnant, unmanipulated, wt animals. The results of previous research did not provide us with clear predictions of the outcomes of these transplantation experiments. For example, the PR is expressed in both stromal and epithelial compartments of the mammary gland (4). Within the epithelium, the distribution of the PR is variegated (5). Together, such observations provided no clear indication of the contributions of various subtypes of stromal and epithelial cells to mammary epithelial morphogenesis occurring in the presence or absence of the PR.

By grafting $PR^{-/-}$ epithelium or stroma in combination with PR wt stroma or epithelium, we have found that the primary target for progesterone is the mammary epithelium, while a direct response of the mammary stroma is not required in order for side-branching and lobuloalveolar development to occur. Furthermore, $PR^{-/-}$ mammary epithelial cells can give rise to alveoli when placed in close vicinity to PR wt epithelial cells, indicating that progesterone does not need to act directly on the alveolar cells and instead can orchestrate the morphogenetic and proliferative events of alveologenesis by affecting nearby cells in the mammary epithelium.

MATERIALS AND METHODS

Mice. ROSA26 and $RAG1^{-/-}$ mice were purchased from The Jackson Laboratory. The PR mutant mice were described

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PR, progesterone receptor; wt, wild-type; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; MEC, mammary epithelial cell.

‡To whom reprint requests should be addressed. e-mail: weinberg@wi.mit.edu.

elsewhere (2); transcription of both A and B forms of the PR was disrupted. All mice were bred in 129SV/C57BL6 genetic background.

For PR genotyping, genomic DNA was isolated from tails and analyzed by PCR. PCR was performed by denaturing the DNA at 94°C for 1 min, followed by 30 cycles of amplification: 94°C for 1 min, 60°C for 2 min, 72°C for 1 min, and a final extension step at 72°C for 5 min. The following PR-specific primers were used: *P1* (5′-TAG ACA GTG TCT TAG ACT CGT TGT TG-3′), *P2* (5′-AGC AGA AAA CCG TGA ATC TTC-3′), and a *neo* gene-specific primer, *N2* (5′-GCA TGC TCC AGA CTG CCT TGG GAA A-3′).

Presence of the β-galactosidase transgene was tested for by subjecting a piece of tail to the 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) staining procedure described below.

Whole-Breast Transplant. Four- to 6-week-old $PR^{+/+}$ or $PR^{-/-}$ female mice were sacrificed and their inguinal mammary glands were dissected. $RAG1^{-/-}$ females of the same age were anesthetized with Avertin i.p. (6). The ventral skin was incised and the abdominal muscle wall was exposed. A $PR^{-/-}$ and a $PR^{+/+}$ mammary gland were placed onto the abdominal wall and the incision was closed with surgical staples. Three weeks after surgery the recipients were mated. They were sacrificed at parturition. The two transplanted glands and an endogenous mammary gland were analyzed by whole-mount microscopy.

Fat-Pad Transplant. Three-week-old $PR^{+/+}$, $PR^{+/-}$, and $PR^{-/-}$ females were sacrificed and their inguinal mammary glands were exposed. The nipple-near region was removed. Into the remaining empty fat pad we injected primary mammary epithelial cells derived from ROSA26 females. The engrafted fat pads were placed onto the abdominal muscle wall of virgin $RAG1^{-/-}$ females.

Transplantation of Mammary Epithelium. The fat pads of 3-week-old $RAG1^{-/-}$ females were cleared (see above). Pieces of mammary tissue of 1-mm diameter were removed from the nipple region of $PR^{+/+}$ and $PR^{-/-}$ females and implanted as described before (7). Alternatively, the cleared fat pads were injected with $PR^{+/+}$ and $PR^{-/-}$ primary cells, cultured as described in ref. 8.

Mammary Gland Whole Mounts. The inguinal mammary glands were dissected, spread onto a glass slide, fixed in a 1:3 mixture of glacial acetic acid/100% ethanol, hydrated, stained overnight in 0.2% carmine (Sigma) and 0.5% $AlK(SO_4)_2$, dehydrated in graded solutions of ethanol, and cleared in 1:2 benzyl alcohol/benzyl benzoate (Sigma) as described previously (9).

Pictures were taken on a Leica MZ12 stereoscope with Kodak Ektachrome 160T.

X-Gal Staining. The transplanted mammary glands were dissected, fixed for 1 hr in 4% formaldehyde in phosphate-buffered saline (PBS), washed three times over 3 hr with rinse buffer (2 mM $MgCl_2$/0.1% sodium deoxycholate/0.2% Nonidet P-40 in PBS) and rotated in X-Gal staining solution (1 mg/ml X-Gal, 5 mM potassium ferricyanide, and 5 mM potassium ferrocyanide in rinse buffer) at 37°C for 18 hr, washed in PBS, and processed for whole-mounting as described above.

Histological Examination and Immunohistochemistry. For histological examination of the alveolar structures the whole-mounted mammary glands were washed in 100% ethanol prior to paraffin embedment. Sections were cut at 10 μm. Anti-β-casein antiserum (10) was diluted 1:500 and applied overnight at 4°C. Biotinylated secondary antibodies were detected with a Vectastain ABC kit (Vector Laboratories).

RESULTS

Development of the Mammary Gland During Pregnancy in the Absence of the PR. To analyze the role progesterone plays in the mammary gland during normal pregnancy, entire mammary glands from $PR^{-/-}$ female mice and their wt littermates were transplanted onto the abdominal muscle wall of PR wt females. The transplanted glands included both epithelial and stromal compartments. The recipient females were of the same 129SV/C57BL6 genetic background and were homozygous for the inactivated RAG1 allele (11). Females of this genotype are immunocompromised and therefore able to accept allografts. The engrafted females were mated 3 weeks after surgery and sacrificed immediately after a completed pregnancy. In all cases, the implants along with an endogenous mammary gland were analyzed by whole-mount microscopy.

While the wt implants and endogenous glands (Fig. 1 *Center* and *Right,* respectively) showed full alveolar development at parturition, the $PR^{-/-}$ grafts developed only a simple ductal system (Fig. 1 *Left*). These observations validated the transplantation procedure. More significantly, they demonstrated, as suggested by previous reports (1, 12), that progesterone is essential for side-branching and lobuloalveolar growth and showed that, in the absence of the PR, the mammary gland fails to undergo substantial proliferation in the presence of the full array of pregnancy-associated hormones.

Involvement of the Stromal and the Epithelial Compartments in PR-Mediated Responses. To address the question of whether progesterone acts on the mammary stroma or epithelium, engrafted animals were created in which either the mammary epithelium or the fat pad lacked PR because of inactivation of the PR gene. The development of the mammary gland in response to physiological hormonal stimulation was then followed.

In the mouse, the mammary epithelium grows out from the nipple into a fat pad that underlies the skin. At three weeks after birth, the epithelium of the gland has not yet penetrated extensively into the stroma and can be eliminated by removing the nipple region of the mammary gland (7). Mammary epithelial cells (MECs) that are introduced into the remaining "cleared" fat pad will give rise to a new ductal system. They can grow out from a piece of breast tissue that is placed into the fat pad (7, 13), or from single-cell suspensions that are injected into the fat pad (14).

We adapted these surgical procedures to create mammary glands that specifically lacked the PR in their stromal cells. Briefly, the nipple regions containing the mammary epithelium were removed from the fourth mammary glands of 3-week-old $PR^{-/-}$ females and their wt littermates. The resulting cleared fat pads were then implanted with mammary epithelium derived from a wt donor. Subsequently, the resulting reconstituted mammary glands were dissected and transplanted onto the abdominal muscle wall of $RAG1^{-/-}$ females.

We validated this transplantation procedure by implanting PR wt epithelium into PR wt fat pads. The resulting engrafted glands developed like the endogenous mammary glands in virgin as well as postpartum recipients, demonstrating that the

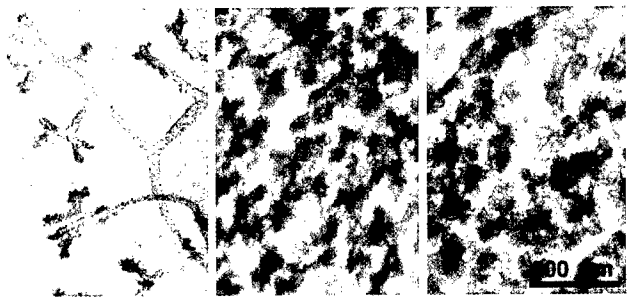


FIG. 1. Whole breast transplantation. Whole-mount preparations of the $PR^{-/-}$ (*Left*) and $PR^{+/+}$ (*Center*) whole breast implant and endogenous mammary gland (*Right*) derived from $RAG1^{-/-}$ recipient mouse after parturition.

engrafted fat pad had become fully vascularized when transplanted in this fashion.

The interpretation of these experiments depended upon our ability to distinguish implanted mammary epithelium from any residual endogenous epithelium that inadvertently had not been removed during the preparation of the cleared mammary fat pads. In fact, in the virgin gland, it is easy to distinguish ducts arising from implanted epithelium from those that are endogenous to this gland because of the distinctive orientations of ductal growth. Thus, the endogenous epithelium grows unidirectionally from the nipple into the fat pad, whereas the ducts arising from the implant, which we place into the center of the cleared fat pad, grow centrifugally. At parturition, however, when the fat pad is filled with alveoli, it is difficult to distinguish the two ductal systems, making it impossible to rule out that the observed epithelial structures derive from residual endogenous epithelium.

To address this difficulty, mammary epithelium derived from ROSA26 female mice was exploited (15). Mice of this transgenic strain express the β-galactosidase gene in virtually all their tissues. The mammary epithelium of these ROSA26 mice was implanted into the cleared fat pads of wt mice. When these reconstituted fat pads were subjected to an X-Gal staining procedure, the implanted ROSA26-derived epithelium turned blue and could thus be unequivocally distinguished from any endogenous epithelium, which was visualized by the red color of the carmine/alum counterstain. Together, the above-described preliminary experiments and the use of ROSA26 cells validated our transplantation procedures and our ability to study engrafted tissues without the confounding effects of residual tissue originating from the recipient breast.

The above procedures were utilized to resolve the respective roles of stroma- and epithelium-derived PR populations in mammary gland proliferation and differentiation. First, ROSA26.PR$^{+/+}$ epithelium was transplanted into cleared PR$^{-/-}$ fat pads; the resulting reconstituted mammary glands were then placed onto the abdominal muscle wall of a RAG1$^{-/-}$ recipient female. Four weeks later, the engrafted RAG1$^{-/-}$ recipients were mated. After they had given birth, the transplanted mammary gland and an endogenous mammary gland were analyzed by whole-mount microscopy. As can be seen in Fig. 2, the injected ROSA26-derived mammary epithelial cells grew equally well in transplanted fat pads from wt (Fig. 2 *Right*) and PR$^{-/-}$ (Fig. 2 *Left*) donors. This result demonstrated that the presence of the PR in the mammary stroma was not essential for the pregnancy-induced side-branching and lobuloalveolar development.

Next, we assessed the role of the PR in the epithelium independent of its function in the stroma. To do this, mammary epithelial cells derived from either PR$^{-/-}$ or wt donors were transplanted into the cleared mammary fat pads of wt recipients. The engrafted recipients were mated and their mammary glands were analyzed at parturition. The results of these experiments are shown in Fig. 3. Whereas the wt implant gave rise to a fully developed mammary tree, the epithelium lacking the PR grew into only a simple ductal tree (Fig. 3 *Left*). Similarly, when we analyzed the mammary glands of engrafted virgin females 2 months after surgery, the wt implant as well as the endogenous breasts showed side-branching, whereas the PR$^{-/-}$ breast had only a simple ductal system (Fig. 3 *Right*). Table 1 summarizes the results of these transplantation experiments. These results allowed us to conclude that the mammary epithelium is the prime target of progesterone both before and during pregnancy, and that a direct response of the mammary stroma to progesterone does not play an essential role.

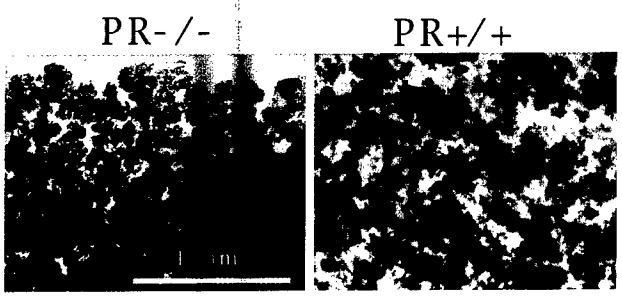


FIG. 2. Transplantation of engrafted fat pads. Whole-mount preparations of transplanted reconstituted breasts. Fat pads from PR$^{-/-}$ or PR$^{+/+}$ mice were engrafted with ROSA26 (β-galactosidase$^+$) PR$^{+/+}$ primary mammary epithelial cells and transplanted onto the abdominal muscle wall of PR$^{+/+}$.RAG1$^{-/-}$ recipients. the reconstituted mammary glands were removed from the recipients after parturition and stained with X-Gal before whole-mounting.

Role of the PR in the Development of Alveoli. The experiments above indicated that the absence of the PR from all cells of the mammary epithelium resulted in a failure of side-branching and lobuloalveolar growth. However, they did not address the question of whether the presence of PR was required in all cells of the ductal epithelium or in only a subset of MECs in order for these morphogenetic processes to proceed normally.

To distinguish between these possibilities, we created mosaic mammary epithelia containing both PR$^{-/-}$ and PR$^{+/+}$ MECs. The latter cells were derived from ROSA26 mice. In this case, tissue structures composed of PR$^{+/+}$ cells would turn blue upon X-Gal staining when analyzed by whole-mount microscopy. Structures composed of PR$^{-/-}$ cells would turn red, being stained only by the carmine/alum counterstain.

Mixtures of PR$^{+/+}$ and PR$^{-/-}$ MECs in different ratios were injected into the cleared mammary fat pads of RAG1$^{-/-}$ females. These mixtures were obtained either by combining single-cell suspensions derived from PR$^{-/-}$ and PR$^{+/+}$.ROSA26 primary cultures or by mixing finely minced mammary tissues dissected from females of these two strains. Two months later, the engrafted recipients were mated, and the engrafted breasts were analyzed toward the end of pregnancy.

Depending on the degree of homogeneity of the injected mixture and the ratio in which the cells of the different genotypes were mixed, we found two types of chimerism. In the

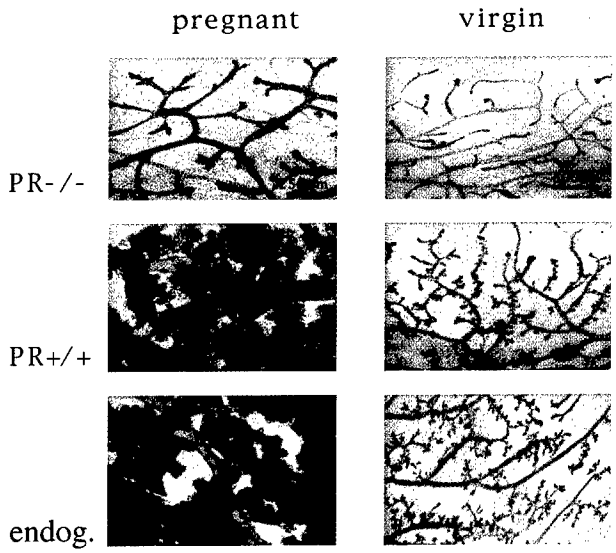


FIG. 3. Transplantation of epithelium. Whole-mount preparations of mammary glands from PR$^{+/+}$.RAG1$^{-/-}$ recipients. (*Left*) Preparation derived from a recipient after parturition. (*Right*) Preparation derived from a virgin mouse. (*Top*) Transplanted PR$^{-/-}$ epithelium. (*Middle*) Transplanted PR$^{+/+}$ epithelium. (*Bottom*) Endogenous mammary gland.

Table 1. Requirement of the PR in the stroma and/or the epithelium for alveolar development in mammary transplants analyzed post partum

Transplant	No. samples with alveolar growth/no. successful transplants
Mammary glands *in toto*	
Stroma $PR^{+/+}$/epithelium $PR^{+/+}$	8/8
Stroma $PR^{-/-}$/epithelium $PR^{-/-}$	0/8
Fat pad injected with $PR^{+/+}$ROSA26 epithelium cells	
Stroma $PR^{+/+}$/injected epithelium $PR^{+/+}$	6/6
Stroma $PR^{+/-}$/injected epithelium $PR^{+/+}$	8/8
Stroma $PR^{-/-}$/injected epithelium $PR^{+/+}$	6/6
Epithelium	
Stroma (host) $PR^{+/+}$/epithelial transplant $PR^{+/+}$	13/13
Stroma (host) $PR^{+/+}$/epithelial transplant $PR^{-/-}$	0/13

first type, the mammary glands showed discrete sectors having distinct phenotypes. An example, representative of 17 samples of this type of chimerism, is shown in Fig. 4. One half of the epithelial component of the mammary gland stained red while the other half stained blue; this indicated the origins of these two sectors from $PR^{-/-}$ and ROSA26 engrafted cells respectively. The sector composed of the $PR^{-/-}$ cells represents a simple ductal tree, whereas the sector composed of the $PR^{+/+}$.ROSA26 cells shows extensive lobuloalveolar growth. This result demonstrated that the coexistence of MECs of $PR^{+/+}$ and $PR^{-/-}$ in one fat pad is not sufficient to rescue the morphogenetic defect intrinsic to the $PR^{-/-}$ cells.

Most of the chimeric epithelia that arose from single-cell suspensions in which the wt cells were in 10-fold excess over $PR^{-/-}$ cells showed complete lobuloalveolar development. However, at higher magnification distinct red alveoli and blue alveoli could be identified. This observation suggested but did not prove that $PR^{-/-}$ cells could participate in alveolar formation if they were in close proximity with wt MECs.

Any conclusions concerning the ability of the $PR^{-/-}$ MECs to form alveoli were clouded by the possibility that certain $PR^{+/+}$.ROSA26 cells that participated in alveologenesis had failed to stain blue, thereby taking on the appearance of the $PR^{-/-}$ cells in the same mixed grafts. To address this issue, we crossed the β-galactosidase transgene into the $PR^{-/-}$ genetic background. By transplanting $PR^{-/-}$.ROSA26 mammary epithelium into wt recipients and analyzing the transplanted glands after birth we were assured that the transgene did not affect the $PR^{-/-}$ phenotype (data not shown). Subsequently, suspensions of $PR^{-/-}$.ROSA26 MECs were mixed with $PR^{+/+}$ MECs lacking the β-galactosidase transgene to generate chimeric breasts. On this occasion, we looked for a result opposite to that seen previously—alveolar cells that stained blue. Indeed, as shown in Fig. 4 *Center*, a representative of 26 independent grafts, the mammary glands obtained from pregnant engrafted females showed areas with blue alveoli, proving conclusively that $PR^{-/-}$ cells can participate in the formation of alveoli if they are in close vicinity to wt epithelial cells.

To determine whether the alveolar structures constituted by $PR^{-/-}$ cells are functional we assessed their morphology on histological sections. As shown in Fig. 4 *Right*, the lumina of the blue $PR^{-/-}$ alveoli compare with those of wt alveoli, indicating the presence of secreted material. Similarly, secretory vacuoles are present. Immunostaining with anti-β-casein antibody revealed the expression of the milk protein (arrow, Fig. 4 *Upper Right*). Together these results indicate that the $PR^{-/-}$ alveoli are fully differentiated. Thus, the presence of the PR is required in only a portion of the MECs in order for lobuloalveolar development to occur. Moreover, these findings suggest that progesterone activates a paracrine signaling route that operates between distinct subtypes of MECs, permitting $PR^{-/-}$ MECs to participate directly in lobuloalveolar proliferation and differentiation.

DISCUSSION

Hormonal ablation/reconstitution experiments (1) have suggested that progesterone plays an important role in the changes that the mammary gland undergoes during early pregnancy,

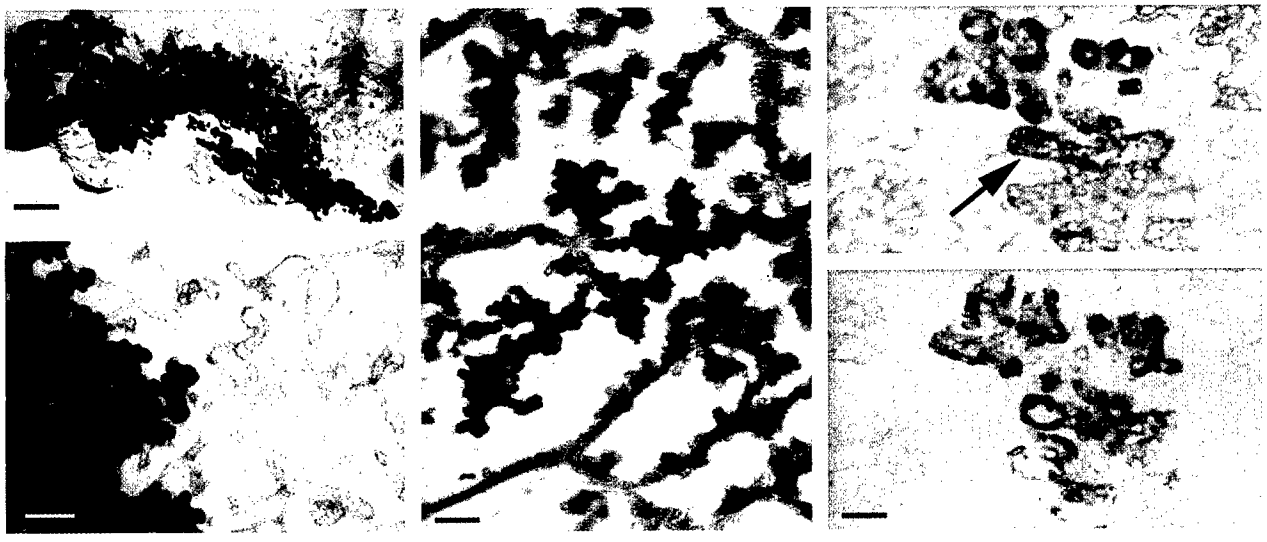


FIG. 4. Rescue of the $PR^{-/-}$ phenotype in $PR^{-/-}$ and $PR^{+/+}$ chimeric epithelia. (*Left*) Whole-mount preparation of cleared $PR^{+/+}$.$RAG1^{-/-}$ fat pad implanted with a mixture of $PR^{-/-}$ (red) epithelium and ROSA26.$PR^{+/+}$ epithelium (blue) in a 1:1 ratio. The engrafted mammary gland was removed after the recipient had given birth, subjected to X-Gal staining, and whole-mounted.(Bar in *Upper* corresponds to 2 mm; bar in *Lower*, to 200 μm). (*Center*) Whole-mount preparation of cleared $PR^{+/+}$.$RAG1^{-/-}$ fat pad injected with a mixture of $PR^{-/-}$.ROSA26 (blue) epithelium and $PR^{+/+}$ epithelium (red) injected in a 1:10 ratio, treated as for *Left*. (Bar corresponds to 200 μm.) (*Right*) Adjacent histological sections of an area with $PR^{-/-}$.ROSA26 alveolar structures. (*Upper*) Expression of β-casein in wt and $PR^{-/-}$.ROSA26 alveoli. (*Lower*) Control without primary antibody. Arrow indicates $PR^{-/-}$.ROSA26 alveolus expressing β-casein. (Bar corresponds to 50 μm.)

namely side-branching and initial alveolar growth. To determine the extent to which progesterone signaling is limiting in development, we generated mice lacking the PR gene (2). However, because the $PR^{-/-}$ females have multiple impairments in their reproductive functions, the specific consequences of PR inactivation on mammary gland development could not be assessed in these mice.

To circumvent this difficulty, we have used various transplantation techniques to elucidate the role of progesterone in the development of the mammary gland. In particular, we have made use of cells derived from mice carrying the β-galactosidase transgene. These cells turn blue upon X-Gal staining, making it possible to distinguish these cells histochemically from neighboring β-galactosidase-negative cells. In one experiment, this allowed us to distinguish the β-galactosidase-positive implanted MECs from the β-galactosidase-negative endogenous cells of an engrafted breast; in another setting, this procedure made it possible for us to distinguish MECs carrying two functional PR alleles from those lacking the PR.

Most transplantation experiments involving nonsyngeneic grafts have exploited nude mice as recipients. We note here in passing the utility of the $RAG1^{-/-}$ mice used for transplantation experiments designed to elucidate mammary gland physiology. Because nude mice have low estrogen levels, they do not represent good recipients in transplantation experiments designed specifically to gauge mammary function. In contrast, the $RAG1^{-/-}$ mice used here exhibit developmental defects that are strictly limited to B and T cell development (11).

Our initial experiments involving the transplantation of $PR^{-/-}$ mammary glands into $PR^{+/+}.RAG1^{-/-}$ females were motivated by the need to assess the role of the PR in an *in vivo* physiologic environment in which the full array of pregnancy-associated hormonal signals was present. $PR^{-/-}$ mammary glands grafted into a $PR^{+/+}.RAG1^{-/-}$ recipient developed only a simple ductal system, even when the host went through a series of estrous cycles and a normal pregnancy. This indicated that side-branching and lobuloalveolar growth rely on the presence of the PR, and that other signaling mechanisms operating in the breast tissue cannot compensate for the absence of the PR to allow these processes to proceed normally.

These initial results left us with two distinct scenarios. In one, both side-branching and lobuloalveolar proliferation, each in its own right, depends on the presence of progesterone. In the other, side-branching depends on progesterone, whereas lobuloalveolar growth depends on prior side-branching and is therefore only indirectly dependent on progesterone. Our analysis of a series of whole mounts of mammary glands from wt pregnant mice showed that alveoli sprouted not only from side branches (secondary ducts) but also from the primary ducts (data not shown). This finding indicated that side-branching is not an absolute prerequisite for alveolar growth. For this reason, we concluded that the PR is required for lobuloalveolar proliferation *per se* in addition to its demonstrated role in side-branching.

We next addressed the issue of whether progesterone needs to act on the mammary stroma, the epithelium, or both. One important clue for resolving this puzzle appeared to come from the longstanding observation that morphogenesis in many epithelial-mesenchymal organs such as the mammary gland is controlled by inductive events (16) that require cross-talk between epithelial and stromal components. In the breast in particular, the embryonic mammary mesenchyme induces the overlying epithelium to develop into the mammary bud (17). Moreover, in male embryos of various mouse strains, androgens act on the stroma to induce the involution of the mammary anlage (18, 19). The estrogen receptor is required in the mammary stroma for ductal growth to occur (20).

The role of the stroma in mediating progesterone-dependent processes in the breast has been less clear. For example, ligand-binding studies have shown that 80% of the progesterone receptors in the mouse mammary gland localize to the epithelium, while the remaining 20% are found in the stroma (4). Such observations have been compatible with models in which the epithelial cells, the stromal cells, or both cell types are required to mediate the direct responses to progesterone.

More recently, epithelial/stromal reciprocal transplantations between wt and estrogen receptor $(ER)^{-/-}$ and wt and $PR^{-/-}$ tissues have demonstrated that stromal derived ER and PR exert paracrine effects on the epithelium both in the uterus (21) and in the vagina (G. R. Cunha and B.W.O., unpublished observations). We show here that mammary glands lacking PR in the stroma undergo normal development, whereas the absence of the PR from the epithelium confers the $PR^{-/-}$ phenotype, indicating that the target cells of progesterone in the mammary gland are in the epithelium. While effects of progesterone on the mammary stroma cannot be excluded, they do not appear to contribute in any obvious way to the development of the ductal tree and alveoli.

Recently reported experiments in which we participated (3) yielded results that are in conflict with one aspect of the present work. These previous experiments appeared to indicate that the PR that functions within the stromal compartment exerts an effect on epithelial ductal growth, contrary to the present results, which indicate the opposite. We find the present results more compelling for several reasons. The number of transplanted animals examined here was much larger. Moreover, we have analyzed the behavior of mammary glands in a situation in which the only PR-negative tissue in engrafted animals was the mammary stroma; the earlier work, in contrast, examined the behavior of wt epithelium transplanted into the cleared $PR^{-/-}$ fat pad of a $PR^{-/-}$ host. In concordance with our conclusion, a recent immunostaining failed to detect any PR protein in the fat pad (22).

The present work together with previous observations of others (1, 12) indicates that progesterone is required for two distinct morphogenetic processes in the breast—side-branching and preparation of ductal cells for subsequent lobuloalveolar development. The precise mechanisms by which progesterone enables ductal MECs to participate in alveologenesis has been unclear. The pattern of PR expression in the mammary epithelium is inhomogeneous (5), suggesting the involvement of only a subset of ductal cells in progesterone-triggered processes. The connected issue of whether the PR-expressing cells represent the precursors of the alveolar outgrowths is addressed here.

Our observation that $PR^{-/-}$ cells can give rise to alveolar structures if they are in close vicinity to $PR^{+/+}$ cells indicates that progesterone does not need to act directly on a ductal epithelial cell for it to participate in alveolar formation. Instead, it appears that progesterone acts on a subtype of ductal cell, causing it to release paracrine signals that permit other nearby epithelial cells to participate directly in lobuloalveolar proliferation.

The present work provides no indication about the nature of the paracrine signal released by the progesterone-activated ductal cell. However, the observation that close apposition of PR-positive with PR-negative cells is required to rescue the $PR^{-/-}$ phenotype indicates that the signal, whatever its biochemical nature, is transmitted only over short intercellular distances. Factors that are tightly associated with the extracellular matrix such as wnt proteins and fibroblast growth factors, which are differentially expressed during mammary gland development (23, 24), are attractive candidates for conveying such paracrine signals.

Our data provide no indication whether or not these paracrine signals communicate directly between the progesterone-

activated ductal cells and closely apposed alveolar precursor cells. It remains equally possible that the progesterone-activated ductal cell communicates with the stroma; the latter, in turn, may pass on a signal directly to the alveolar precursor cells as suggested by others (25). The use of tissue reconstitution techniques and genetically altered cells should allow the further dissection of the molecular mechanisms of mammary morphogenesis over the next several years.

We thank Ms. Frances Kittrell and Dr. Daniel Medina for continued advice, Ms. Gouqingge for technical assistance, Dr. Ernst Reichmann for the generous gift of β-casein antiserum and the Dr. Mildred-Scheel foundation for their support. This work was supported by grants from the Department of the Army, Breast Cancer Research Program, the G. Harold & Leila Y. Mathers Charitable Foundation, and National Cancer Institute Grant OIG R35CA39826.

1. Nandi, S. (1958) *J. Natl. Cancer Inst.* **21,** 1039–1063.
2. Lydon, J. P., De Mayo, F. J., Funk, C. R., Mani, S. K., Hughes, A. R., Montgomery, C. A., Jr., Shyamala, G., Conneely, O. M. & O'Malley, B. W. (1995) *Genes & Dev.* **9,** 2266–2278.
3. Humphreys, R., R., Lydon, J., O'Malley, B. W. & Rosen, J. M. (1997) *Mol. Endocrinol.* **11,** 801–811.
4. Haslam, S. Z. & Shyamala, G. (1981) *Endocrinology* **108,** 825–830.
5. Silberstein, G. B., Van Horn, K., Shyamala, G. & Daniel., C. W. (1996) *Cell Growth Differ.* **7,** 945–952.
6. Hogan, B. L. M., Beddington, R., Constantini, F. & Lacy, E. (1995) *Manipulating the Mouse Embryo: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, New York), 2nd Ed.
7. DeOme, K. B., Faulkin, L. J., Jr., Bern, H. A. & Blair, P. B. (1959) *Cancer Res.* 511–520.
8. Kittrell, F. S., Oborn, C. J. & Medina, D. (1992) *Cancer Res.* **52,** 1924–1932.
9. Wang, S., Counterman, L. J. & Haslam, S. Z. (1990) *Endocrinology* **127,** 2183–2189.
10. Reichmann, E., Groner, B. & Friis, R. R. (1989) *J. Cell Biol.* **108,** 1127–1138.
11. Mombaerts, P., Iacomini, J., Johnson, R. S., Herrup, K., Tonegawa, S. & Papaioannou, V. E. (1992) *Cell* **68,** 869–877.
12. Lyons, W. R. (1958) *Proc. R. Soc. London Ser. B* **149,** 303–325.
13. Daniel, C. W., Shannon, J. M. & Cunha, G. R. (198) *Mech. Ageing Dev.* **23,** 259–264.
14. Daniel, C. W. & DeOme (1965) *Science* **149,** 634–636.
15. Friedrich, G. & Soriano, P. (1991) *Genes Dev.* **5,** 1513–1523.
16. Grobstein, C. (1955) *J. Exp. Zool.* **130,** 319–340.
17. Propper, A. (1968) *Ann. Embryol. Morphol.* **2,** 151–160.
18. Kratochwil, K. & Schwartz, P. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 4041–4044.
19. Drews, U. & Drews, U.(1977) *Cell* **10,** 401–404.
20. Cunha, G. R., Young, P., Hom, Y. K., Cooke, P. S., Taylor, J. A. & Lubahn, D. B. (1997) *J. Mammary Gland Biol. Neoplasia* **2,** 393–402.
21. Cooke, P. S., Buchanan, D., L., Young, P., Setiawan, T., Brody, J., Korach, K. S., Taylor, J., Lubahn, D. B. & Cunha, G. R. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 6535–6540.
22. Shyamala, G., Barcellos-Hoff, M. H., Toft, D. & Yang, X. (1997) *J. Steroid Biochem. Mol. Biol.* **63,** 251–259.
23. Gavin, B. J. & McMahon, A. P. (1992) *Mol. Cell. Biol.* **12,** 2418-2423.
24. Coleman-Krnacik, S. & Rosen, J. M. (1994) *Mol. Endocrinol.* **8,** 218–229.
25. Birchmeier, C., Sonnenberg, E. & Birchmeier, W. (1993) *BioEssays* **15,** 185–190.